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Mehellou

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Aryloxy Triester Phosphoramidates as Phosphoserine Prodrugs- A Proof of Concept Study

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Abstract: The specific targeting of protein-protein interactions by phosphoserine-containing small molecules has been scarce due to the dephosphorylation of phosphoserine and its charged nature at physiological pH, which hinders its uptake into cells. To address these issues, we herein report the synthesis of phosphoserine aryloxy triester phosphoramidates as phosphoserine prodrugs, which are enzymatically metabolized to release phosphoserine. This phosphoserine masking approach was applied to a phosphoserine-containing inhibitor of 14-3-3 dimerization, and the generated prodrugs exhibited improved pharmacological activity. Collectively, this provided a proof of concept of the masking of phosphoserine with biocleavable aryloxy triester phosphoramidate masking groups as a viable intracellular delivery system for phosphoserine-containing molecules. Ultimately, this will facilitate the discovery of phosphoserine-containing small molecule therapeutics.

Protein phosphorylation at serine residues is a fundamental phenomenon that is used by cells to affect the function, localization and degradation of proteins.^[1] In some cases, phosphorylated serine residues mediate protein-protein interactions via the docking of the phosphoserine residue into a positively charged pocket within the partner protein. An example of this is the serine phosphorylation of the adaptor protein 14-3-3, which facilitates its homodimerization.^[2] Attempts at inhibiting these phosphoserine-mediated protein-protein interactions with small molecules have mostly led, by design, to small molecules that lack phosphoserine. The move to discard phosphoserine groups from these molecules was driven by the fact that phosphoserine carries two negative charges at physiological pH, which limit its (passive) cellular uptake.^[3] Additionally, phosphoserine is also subject to dephosphorylation by alkaline phosphatases, a process that yields serine-containing derivatives that do not often retain potent pharmacological activity compared to their parent phosphoserine-containing molecules. Although the

masking of the phosphate group of phosphoserine was previously investigated, the study was limited to a phosphoserine mimetic (difluoromethylenephosphoserine) and not the natural phosphoserine moiety.^[4] With this in mind and in order to improve the drug-like properties of phosphoserine-containing small molecules, we explored the application of the aryloxy triester phosphoramidate technology to phosphoserine. This technology has been widely used to mask the 5'-O-monophosphate groups of nucleotides, and has so far led to two FDA-approved drugs; sofosbuvir and tenefovir alafenamide.^[5]

As a proof of concept, we initially synthesized phosphoserine with the aryloxy triester phosphoramidate masking groups (**Figure 1a**). For this, the *N*- and *C*-terminals of phosphoserine had to be protected^[6] first to allow for the selective addition of the aryloxy triester phosphoramidate moiety to the side chain hydroxyl group. The synthesis was initiated by the chlorination of *tert*-butanol (**1**) by copper(I) chloride with the peptide coupling reagent *N,N'*-dicyclohexylcarbodiimide (DCC).^[6] The generated compound, **2**, was then reacted with the commercially available *N*-Boc *L*-serine, **3**, in DCM to yield the *N*- and *C*-protected *L*-serine **4**.^[6] This latter compound was subsequently reacted with phenyl *L*-alanine methyl ester phosphorochloridate (**7**), which had been synthesized according to reported procedures^[7] by reacting *L*-alanine methyl ester hydrochloride (**6**) with the commercially available phenyl dichlorophosphate (**5**) in DCM and in the presence of triethylamine (NEt₃). This reaction yielded the desired phosphoserine aryloxy triester phosphoramidate, compound **8**, as a white solid in a good yield (62%).

With the phosphoserine aryloxy triester phosphoramidate in hand, we first studied whether the aryl and amino acid ester groups that mask the phosphate group of phosphoserine could be metabolized *in vitro* to release the unmasked phosphoserine species. It is now well established that the metabolism of the aryloxy triester phosphoramidate moieties is initiated by the cleavage of the ester motif by carboxypeptidase Y to yield

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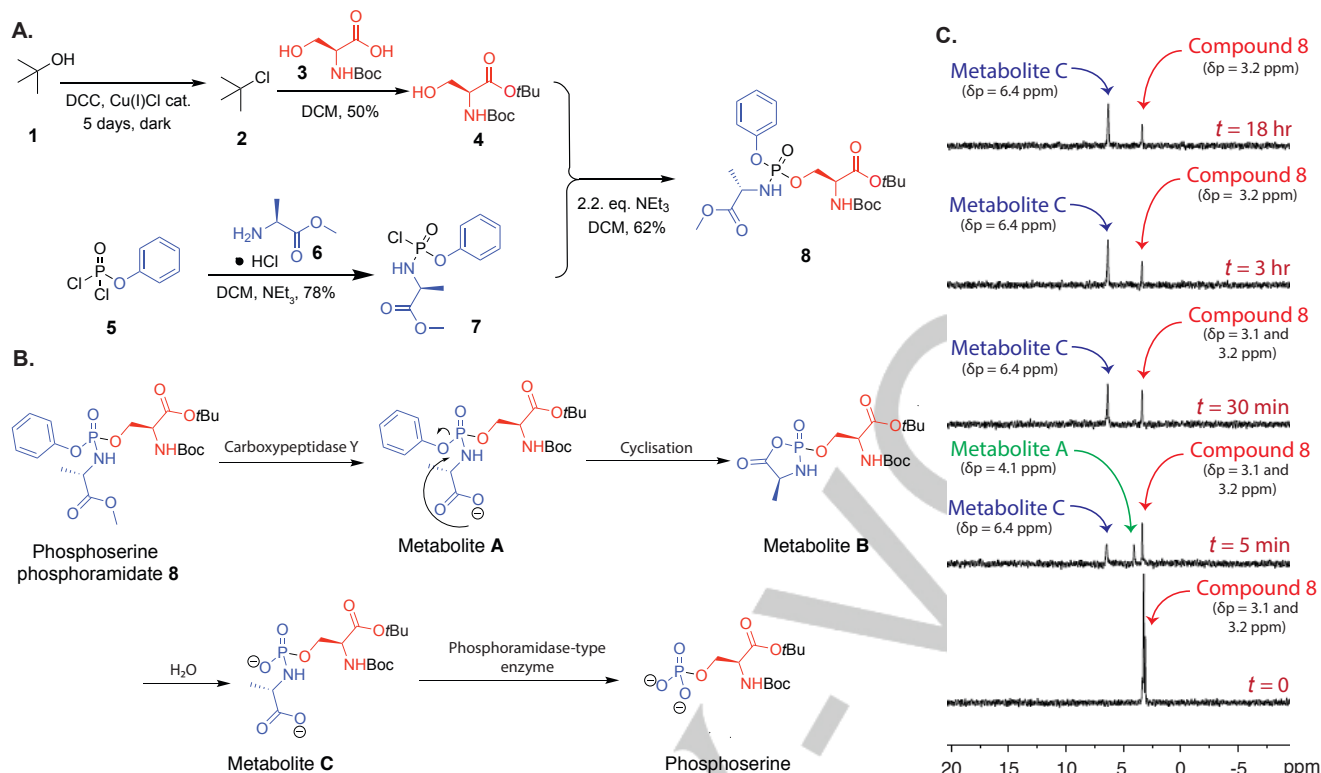


Fig. 1. Synthesis and *in vitro* esterase-mediated metabolism of phosphoserine aryloxy triester phosphoramidates. **A.** Synthesis of a phosphoserine aryloxy triester phosphoramidates (**8**). **B.** Mechanism of aryloxy triester phosphoramidates metabolism. **C.** ³¹P-NMR in vitro enzymatic assay of the breakdown of the phosphoserine phosphoramidate by carboxypeptidase Y.

metabolite **A** (Figure 1b).^[5a, 8] The formed carboxylate group then performs a nucleophilic attack onto the phosphate group leading to the release of the aryl group and the formation of a highly unstable five-membered anhydride ring (metabolite **B**, Figure 1b). This is subsequently opened up by a water molecule to generate the phosphoramidate metabolite **C** (Figure 1b). Finally, the phosphoramidase-type enzyme Hint-1^[9] cleaves the P-N bond of metabolite **C** to release the unmasked monophosphate group. With this in mind, we incubated compound **8** at 37 °C with commercially available recombinant carboxypeptidase Y and monitored the sample by ³¹P-NMR (Figure 1c).

As shown in Figure 1c, at *t* = 0 min, the ³¹P-NMR of compound **8** shows a sharp two singlets (δ_p = 3.1 and 3.2 ppm), which correspond to the two diastereoisomers (*R* and *S*) of the phosphoserine aryloxy triester phosphoramidate. This is typical of the aryloxy triester phosphoramidates as they have a chiral phosphorous and most current synthetic routes yield these compounds as a mixture of two diastereoisomers.^[5a, 7b, 10] After 5 minutes incubation with recombinant carboxypeptidase Y, two new peaks appeared; one appeared briefly at δ_p = 4.1 ppm while another peak at δ_p = 6.4 ppm also appeared and remained throughout the rest of the experiment. This is typical for aryloxy triester phosphoramidates as previous studies using carboxypeptidase Y showed a similar pattern with the peak at δ_p = 4.1 ppm corresponding to metabolite **A** whilst that at δ_p = 6.4 ppm corresponding to metabolite **C** (Figure 1b).^[10-11] Notably, after 30 minutes incubation, only the ³¹P-NMR peak at δ_p = 3.2 ppm remained from the two ³¹P-NMR peaks of the parent compound **8**. This indicated that one of the diastereoisomers of phosphoserine phosphoramidate, which has a ³¹P-NMR that corresponds to δ_p = 3.1 ppm, is a better

substrate for the enzyme and thus was processed quicker. From 30 minutes onward, only two ³¹P-NMR peaks persisted; δ_p = 3.2 and 6.4 ppm. After 18 h, the peak at δ_p = 6.4 ppm became the major peak with only one of the peaks from the original phosphoserine phosphoramidate two singlets remained. Mass spectrometry analysis of the products generated from this *in vitro* enzymatic assay after 18 h confirmed the breakdown of the phosphate masking groups and the release of metabolite **C** (Supporting Figure S1). As in this *in vitro* assay, there was no phosphoramidase-type enzyme, metabolite **C** was not further processed to release the fully unmasked phosphoserine species. To predict whether metabolite **C** would be a good substrate for Hint-1, we performed *in silico* docking of the metabolite into the crystal structure of the human Hint-1 co-crystallized with AMP (PDB 1KPF) as previously reported.^[10] The results showed that metabolite **C** sits in the Hint-1 active site and the phosphate group of phosphoserine forms key interactions with catalytic residues of Hint-1 (serine107, histidine112 and histidine114) suggesting that it could be a good substrate for this enzyme (Supporting Figure S2).^[9b] These docking results are in line with previous docking studies for the same enzyme that predicted and verified whether the P-N bond of the docked substrates could be cleaved off by Hint-1.^[9b] Together, these results indicate that phosphoserine aryloxy triester phosphoramidates are metabolized in a similar fashion to the nucleoside monophosphate aryloxy triester phosphoramidates.

In order to establish the applicability of the aryloxy triester phosphoramidate approach in improving the pharmacological activity of phosphoserine-containing compounds, we applied this approach to a 14-3-3

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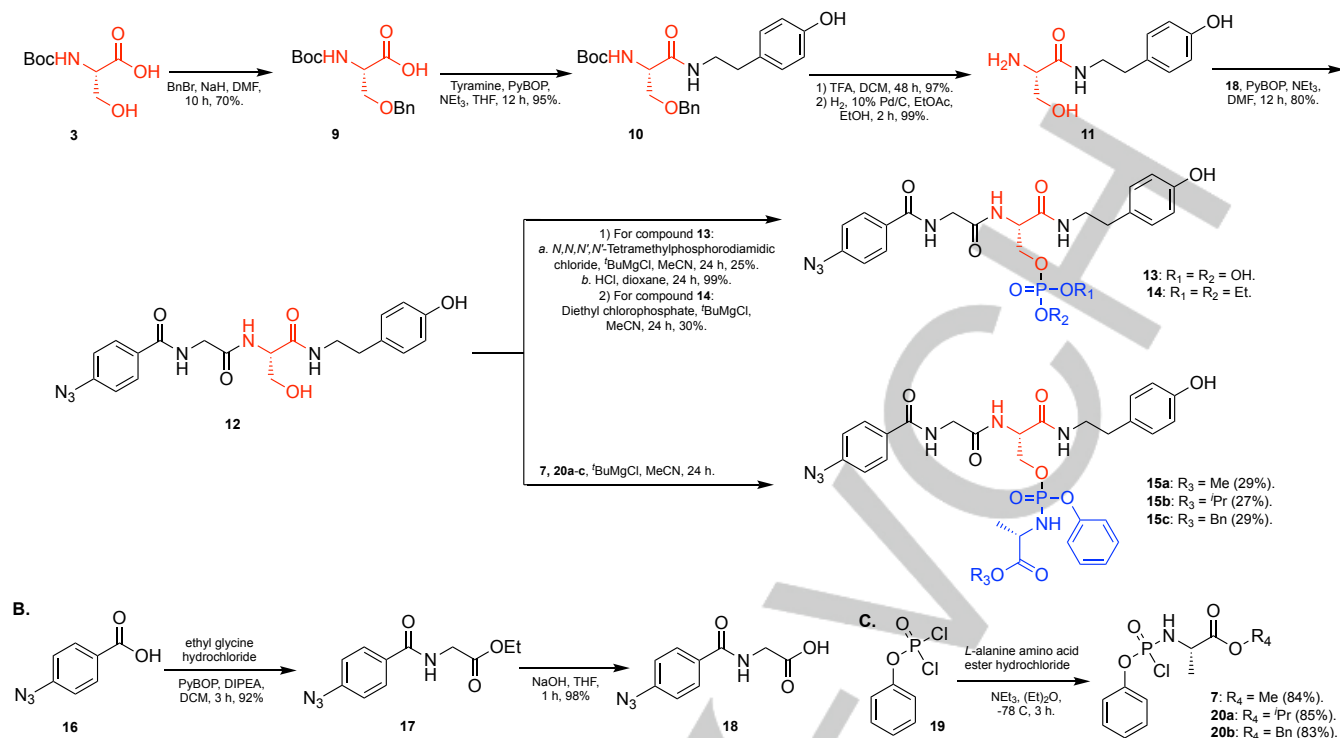


Fig. 2. A. Synthesis of the phosphoserine-containing 14-3-3 dimerization inhibitor (**13**), diethyl phosphate (**14**) and its aryloxy triester phosphoramidates (**15a-c**). The serine motif is shown in red while its phosphate and masked phosphate groups are shown in blue. B. Synthesis of the intermediate 2-((4-azidobenzoyl)oxy)acetic acid (**18**). C. Synthesis of phenyl *L*-alanine ester phosphorochloridates (**7** and **20a-b**).

dimerization inhibitor **13** (Figure 2), which showed promising, but not potent activity in cells.^[12] This compound is an ideal candidate for the application of the aryloxy triester phosphoramidate technology as it contains a phosphoserine motif that is essential for its pharmacological activity.^[12]

The synthesis of the aryloxy triester phosphoramidates of compound **13** commenced by the regioselective benzylation of the side chain hydroxyl group of *N*-Boc-*L*-serine (**3**), which proceeded with 70% yield (Figure 2a). The carboxylic acid of the product, **9**, was then conjugated to the amine group of tyramine to form a peptide bond using the peptide coupling reagent PyBOP in THF in the presence of NEt₃. This was then followed by sequential deprotection of the amine group and the side chain hydroxyl moiety using trifluoroacetic acid in DCM and hydrogenation, respectively. This resultant compound **11** was reacted with compound **18**, which had been generated^[13] in two steps from the commercially available 4-azidobenzoic acid (Figure 2b), to generate compound **12**.

For the synthesis of the reported 14-3-3 dimerisation inhibitor, **13**, compound **12** was reacted with *N,N,N',N'*-tetramethylphosphorodiamidic chloride in MeCN in the presence of the Grignard reagent *tert*butyl magnesium chloride (^tBuMgCl). The generated phosphorodiamidate derivative was then converted to the desired monophosphate species, **13**, by treatment with HCl in dioxane. We also synthesized the derivative of compound **13** where the phosphate group is fully masked by diethyl groups, compound **14**, to be used as a negative control in the biological assays. The synthesis was achieved by reacting compound **12** with diethyl chlorophosphate in the presence of ^tBuMgCl in MeCN to flourish the desired compound **14** in 30% yield. For the synthesis of the aryloxy triester

phosphoramidates of the 14-3-3 dimerization inhibitor, **13**, the synthesis was accomplished by coupling compound **12** to the appropriate phosphorochloridate (**7**, **20a** or **20b**, Figure 2c) in the presence of ^tBuMgCl in MeCN to give the desired aryloxy triester phosphoramidates (**15a-c**), as a mixture of two diastereoisomers, in low yields (~ 30%). Notably, the phosphorochloridates **7**, **20a-b** were synthesised as shown in Figure 2c and reported previously.^[7]

In order to determine the efficacy of the synthesized aryloxy triester phosphoramidate prodrugs to inhibit 14-3-3 dimerization and cell proliferation, we treated the lung cancer cell line, A549, with the 14-3-3 dimerization inhibitor, **13**, its diethyl phosphate derivative, **14**, and the aryloxy triester phosphoramidates **15a-c** as well as compound **12** at the indicated concentrations (12.5, 25, 50 and 100 μM) for 48 and 72 h (Figure 3). Cell viability was then determined using standard MTT assays. The results showed that, as expected, the unphosphorylated compound **12**, its phosphorylated derivative **13** and the diethyl phosphate compound **14**, did not show any significant effect on cell viability. Encouragingly, the aryloxy triester phosphoramidates **15a-c**, apart from **15a**, did show significant dose-dependent reduction of A549 cell viability (Figure 3). This is because these aryloxy triester phosphoramidates are neutral at physiological pH, unlike the parent compound **13**, and thus they are more readily taken up by cells via passive diffusion. The fact that among this series of aryloxy triester phosphoramidates, the isopropyl and benzyl ester phosphoramidates (**15b** and **15c**, respectively) showed significant biological activity and the methyl ester phosphoramidate, **15a**, did not demonstrate profound biological activity, is in line with the established structure-

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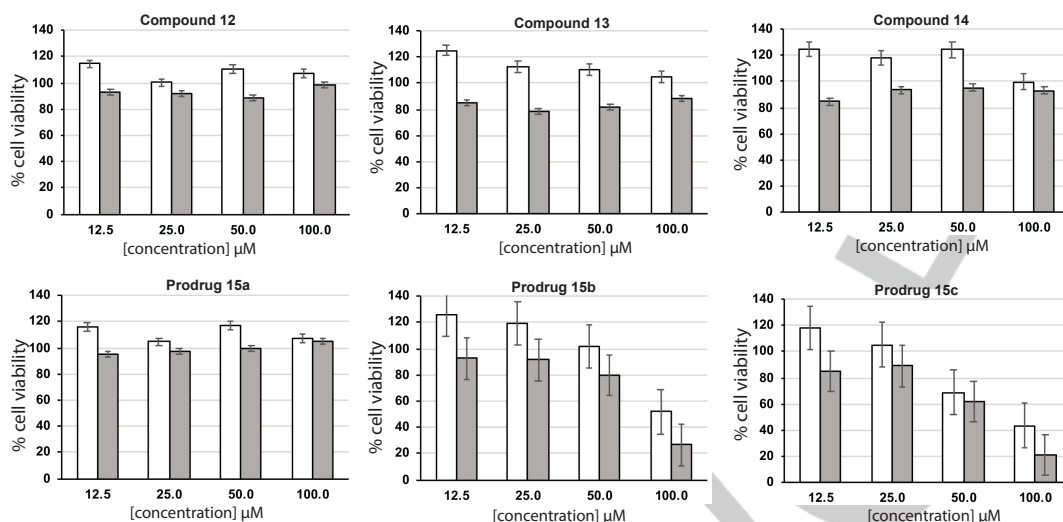


Fig. 3. Cell viability of the unmasked phosphoserine compound (**12**), phosphoserine-containing 14-3-3 dimerization inhibitor (**13**), its diethyl phosphate derivatives (**14**) and the aryloxy triester phosphoramidates (**15a-c**). Cell viability was determined by standard MTT assay. The compounds were incubated with A549 lung cancer cell line for 48 h (white bar) and 72 h (grey bar) at the indicated concentrations. The percentage of cell viability was calculated and presented as normalized value to control DMSO. Error bars show standard error from triplicate experiments.

activity relationship of aryloxytriester phosphoramidates of 5'-O-nucleoside monophosphates.^[5a, 7b]

In summary, masking the phosphate group of phosphoserine with an aryl motif and an amino acid ester serves as a useful approach for the intracellular delivery of phosphoserine. Indeed, this work showed using a combination of *in vitro* enzymatic and *in silico* docking studies that these phosphoserine aryloxy triester phosphoramidates are metabolized to release the unmasked phosphoserine moiety. The application of this approach to a 14-3-3 phosphoserine-containing molecule significantly improved its pharmacological efficacy in cancer cells. Together, this validates this approach of phosphoserine masking with biocleavable motifs, and this will in the future facilitate the discovery of phosphoserine-containing molecules as powerful tool compounds and potential therapeutics.

Experimental Section

Experimental details are found in the Supporting Information.

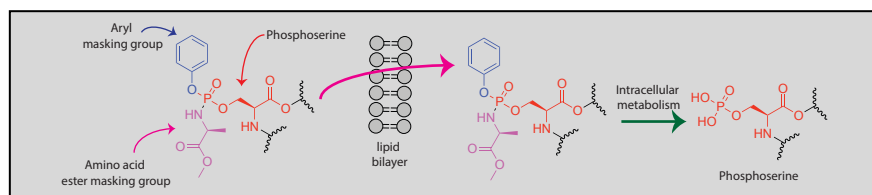
Keywords: Phosphoserine • Phosphoramidate • Prodrug • 14-3-3 • Cancer

- [1] a) T. Pawson, J. D. Scott, *Trends Biochem. Sci.* **2005**, 30(6), 286-290; b) T. Hunter, *Cell* **1995**, 80(2), 225-236; c) P. Cohen, *Trends Biochem. Sci.* **1992**, 17(10), 408-413.
- [2] M. B. Yaffe, K. Rittinger, S. Volinia, P. R. Caron, A. Aitken, H. Leffers, S. J. Gamblin, S. J. Smerdon, L. C. Cantley, *Cell* **1997**, 91(7), 961-971.
- [3] L. Xie, S. Y. Lee, J. N. Andersen, S. Waters, K. Shen, X. L. Guo, N. P. Moller, J. M. Olefsky, D. S. Lawrence, Z. Y. Zhang, *Biochemistry* **2003**, 42(44), 12792-12804.
- [4] A. Arrendale, K. Kim, J. Y. Choi, W. Li, R. L. Geahlen, R. F. Borch, *Chem. Biol.* **2012**, 19(6), 764-771.
- [5] a) Y. Mehellou, H. S. Rattan, J. Balzarini, *J. Med. Chem.* **2018**, 61(6), 2211-2226; b) Y. Mehellou, *ChemMedChem* **2016**, 11(11), 1114-1116.
- [6] S. Koch, D. Schollmeyer, H. Lowe, H. Kunz, *Chemistry* **2013**, 19(22), 7020-7041.

- [7] a) M. S. Davey, R. Malde, R. C. Mykura, A. T. Baker, T. E. Taher, C. S. Le Duff, B. E. Willcox, Y. Mehellou, *J. Med. Chem.* **2017**; b) L. Osgerby, Y. C. Lai, P. J. Thornton, J. Amalfitano, C. S. Le Duff, I. Jabeen, H. Kadri, A. Miccoli, J. H. R. Tucker, M. M. K. Muqit, Y. Mehellou, *J. Med. Chem.* **2017**, 60(8), 3518-3524.
- [8] a) G. Birkus, R. Wang, X. Liu, N. Kutty, H. MacArthur, T. Cihlar, C. Gibbs, S. Swaminathan, W. Lee, M. McDermott, *Antimicrob. Agents Chemother.* **2007**, 51(2), 543-550; b) J. Balzarini, A. Karlsson, S. Aquaro, C. F. Perno, D. Cahard, L. Naesens, E. De Clercq, C. McGuigan, *Proc. Nat. Acad. Sci. USA* **1996**, 93(14), 7295-7299.
- [9] a) J. Cheng, X. Zhou, T. F. Chou, B. Ghosh, B. Liu, C. R. Wagner, *Bioorg. Med. Chem. Lett.* **2009**, 19(22), 6379-6381; b) E. Murakami, T. Tolstykh, H. Bao, C. Niu, H. M. Steuer, D. Bao, W. Chang, C. Espiritu, S. Bansal, A. M. Lam, M. J. Otto, M. J. Sofia, P. A. Furman, *J. Biol. Chem.* **2010**, 285(45), 34337-34347.
- [10] Y. Mehellou, R. Valente, H. Mottram, E. Walsby, K. I. Mills, J. Balzarini, C. McGuigan, *Bioorg. Med. Chem.* **2010**, 18(7), 2439-2446.
- [11] M. Slusarczyk, M. H. Lopez, J. Balzarini, M. Mason, W. G. Jiang, S. Blagden, E. Thompson, E. Ghazaly, C. McGuigan, *J. Med. Chem.* **2014**, 57(4), 1531-1542.
- [12] H. Wu, J. Ge, S. Q. Yao, *Angew. Chem.* **2010**, 49(37), 6528-6532.
- [13] H. Garrido-Hernandez, K. D. Moon, R. L. Geahlen, R. F. Borch, *J. Med. Chem.* **2006**, 49(11), 3368-3376.

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The development of phosphoserine-containing therapeutics has been limited by their poor drug-like properties. To address this, we applied the aryloxy triester phosphoramidate prodrug technology to phosphoserine and a phosphoserine-containing 14-3-3-dimerization inhibitor. These prodrugs exhibited improved biological activity highlighting the promise of this prodrug technology in the discovery of phosphoserine-containing therapeutic.

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